Divergent effects of epidermal growth factor and calcipotriol on human rectal cell proliferation

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Abstract

Vitamin D may protect against colorectal cancer by reducing cell proliferation and inducing differentiation. By contrast, epidermal growth factor (EGF) stimulates cell proliferation and may encourage gastrointestinal mucosal healing. This study investigated the effect of a synthetic vitamin D analogue, calcipotriol, and EGF on human rectal epithelial cell proliferation in patients with familial adenomatous polyposis (FAP). In addition, a new technique to measure the cell cycle time is described. Sigmoidoscopic biopsy specimens were obtained from 14 patients with FAP. Tissue was established in organ culture, with or without the addition of EGF (n=8), or calcipotriol (n=6). Proliferation was determined using (a) metaphase arrest to measure the crypt cell production rate, (b) native mitotic index, and (c) the growth fraction using PC10 antibody. EGF receptor expression was shown using a polyclonal antibody AP12E. Calcipotriol reduced crypt cell production rate by 52% from mean (SEM) 5.29 (1.18) to 2.56 (0.80) cells/crypt/hour ($p<0.01$) and EGF increased crypt cell production rate by 102% from 3.62 (0.59) to 7.33 (0.90) cells/crypt/hour ($p<0.05$), and this tissue expressed the EGF receptor. The growth fraction was 48.40 (4.0)%, and the native mitotic index 1.08 (0.14)%. The cell cycle time was estimated as 94.5 hours and the time for mitosis as one hour. Thus, calcipotriol and EGF have divergent effects on human rectal mucosal proliferation.

(Gut 1994; 35: 1742-1746)

Familial adenomatous polyposis (FAP) has a well recognised genetic basis, which is characterised by the development of numerous adenomas throughout the gastrointestinal tract.¹⁻³ There may also be a hereditary component to the adenomas that occur in family cancer syndrome patients, as well as many cases of sporadic adenomas.⁴⁻⁶ Mutations of the adenomatous polyposis coli (APC) gene are known to cause familial adenomatous polyposis⁷ and the MCC (mutated in colon cancer) gene is mutated and deleted in sporadic colon cancers.⁸ Indeed, the protein structures of the gene products of the MCC and APC gene are thought to be similar.⁹ These findings, together with the increasing acceptance of the adenoma-carcinoma sequence, suggest that patients with FAP are good models for the development of

colorectal cancer in humans.¹⁰⁻¹² Epithelial hyperplasia in the large bowel precedes the development of colorectal cancer in many circumstances¹³⁻¹⁵ and is associated with an increased rectal crypt cell production rate in humans.¹⁶⁻¹⁸

We have previously shown that both calcium and the active metabolite of vitamin D₃, 1,25 (OH)₂ D₃,¹⁹ reduce rectal crypt cell production rate in biopsy specimens taken from patients with FAP.²⁰ The potential therapeutic use of vitamin D is limited by its profound metabolic effects. A synthetic vitamin D analogue calcipotriol (MC-903) retains potent cell regulatory properties while having limited effects on calcium metabolism.²¹⁻²⁴ Indeed, we have previously shown that calcipotriol can reduce in vitro crypt cell production rate by about 50% in tissue taken from normal controls.¹⁹ Synthetic vitamin D₃ analogues with a similar ability to reduce cell proliferation might have a future therapeutic role to control disease in conditions characterised by a hyperproliferative colorectal epithelium.

Epidermal growth factor (EGF) accelerates epithelial proliferation in rat colon^{25,26} and may also stimulate gastrointestinal regeneration and ulcer healing.²⁷⁻²⁹ The EGF peptide binds to the extracellular domain of its receptor,³⁰ and colorectal carcinoma cells express this receptor.³¹ Indeed, malignant colorectal cell lines can respond to EGF by increasing their rate of proliferation, and this may be an EGF stimulated autocrine effect.^{32,33}

We have therefore compared the effect of calcipotriol and EGF on macroscopically normal rectal mucosa obtained from patients with FAP. We have used a stathmokinetic technique to determine crypt cell production rate and immunohistochemistry to estimate the cell cycle time.

Methods

CLINICAL MATERIAL

Paired rectal biopsy specimens were taken from 14 patients with FAP (mean age 42 years, range 29-65), who had previously undergone total abdominal colectomy with ileorectal anastomosis with a mean time from operation of 16 years (range 2-37). All patients were attending regularly for follow up. Specimens were taken between 0900 and 1200 during an outpatient visit. The patients were examined sigmoidoscopically, and a rectal biopsy specimen was obtained from an area of macroscopically normal rectal mucosa. One specimen from each patient was examined

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Accepted for publication
23 March 1994

histologically to exclude the presence of microadenomas, and sections from this tissue were also used for immunohistochemical analysis. The other sample was divided into small pieces, and these explants were used in the organ culture studies. The rectal tissue was cultured with either EGF 10 ng/ml ($n=8$) or calcipotriol 1×10^{-6} M ($n=6$).

CHEMICALS

The synthetic vitamin D analogue calcipotriol (MC-903 in isopropyl alcohol, Leo Pharmaceutical, Ballerup, Denmark) was added to culture medium to produce a stock solution with a final concentration of 1×10^{-6} M MC-903 and was stored at 20°C until use. The control medium was prepared with a similar dilution of alcohol.

Mouse salivary gland epidermal growth factor (Sigma, Poole, UK) was added to organ culture medium to produce a stock solution containing 10 ng/ml of the peptide.

Proliferating cell nuclear antigen, which is expressed by all cells within the proliferative compartment of normal gastrointestinal tissue, was labelled using PC10, a monoclonal antibody directed against proliferating cell nuclear antigen (supplied by Professor David Lane, University of Dundee). AP12E, a rabbit polyclonal antibody directed against EGF receptor, was supplied by Dr W Gullick, RPMS, London.

ORGAN CULTURE AND CRYPT CELL PRODUCTION RATE

Rectal crypt cell production rate was determined by a previously reported and validated technique.^{16 19 20} Briefly, rectal explants were orientated on metal grids within an organ culture dish. In paired studies, the tissue was cultured with or without the addition of either calcipotriol ($n=6$) or EGF ($n=8$). Thus, each patient's tissue acted as its own control. After 15 hours of culture, vincristine 0.6 µg/ml (Oncovin, Eli Lilly, Basingstoke, UK) was added to the culture medium to induce metaphase arrest within rectal crypts. Explants were then removed one, two, and three hours later. Crypt cell production rate was determined after microdissection of at least 20 crypts by plotting the mean number of arrested metaphases per crypt against the time from vincristine administration.³⁴ The slope of this line, determined by least squares linear regression analysis, gave a value for the crypt cell production rate in cells/crypt/hour.^{16 19 20} Crypt cell production rate values were compared using a paired Student's *t* test.

MITOTIC INDEX

Tissue from six patients was immediately fixed in Carnoy's fluid and stored in 70% alcohol. Later, the tissue was rehydrated using serial dilutions of alcohol and was then acid hydrolysed with 1 M HCl for six minutes to 60°C. After staining with Schiff's reagent, the crypts were microdissected and viewed using oil

immersion microscopy. The total number of cells was counted in at least 10 crypts, and the mean number of metaphases was counted in at least 20 crypts per specimen to determine the mean native mitotic index (I_m) expressed as mitoses/1000 cells.

IMMUNOHISTOCHEMISTRY

EGF receptor

To discover if the EGF receptor was expressed in the tissue from the eight patients used to study the effects of EGF, control tissue – that is, tissue not used in the organ culture experiments – was stained with the AP12E polyclonal antibody using an immunoperoxidase method. Immunohistochemical staining was performed on 3 µm thick sections dewaxed in xylene and passed through serial dilutions of alcohol. Endogenous peroxidase activity was blocked with 0.3% H₂O₂ in phosphate buffered saline for 30 minutes. Three sets of slides were then prepared, one test and two control slides. To the first slide anti-EGF receptor rabbit polyclonal antibody (AP12E) was added (170 µg/ml diluted 1:40), to the second slide TRIS buffer was added instead of the primary antibody, and to the third slide the antibody and the free peptide were added (4 µg/ml anti-EGF receptor with 10 ng/ml free peptide). The TRIS buffer and free peptide slides were used as negative controls. Slides were left for one hour and then washed three times with TRIS buffer. A second layer consisting of biotinylated swine antirabbit IgG (1:300 in TRIS buffer) was applied for a period of one hour. After rinsing the slides three times in TRIS buffer, a third layer consisting of a peroxidase conjugated avidin-biotin complex (ABC, Dakopatts, UK), was applied for 45 minutes. The slides were again rinsed three times in TRIS buffer. Lastly, diaminobenzidine solution 250 µg with 0.037% H₂O₂ (to show peroxidase activity) was applied for five minutes, before slides were washed in tap water and counterstained with haematoxylin.^{35 36}

Proliferating cell nuclear antigen

As a further index of cell proliferation, tissue expression of proliferating cell nuclear antigen was sought using the monoclonal antibody PC10. Tissue from the six patients used to determine the mitotic index was fixed in formalin and processed with 24 hours for optimal demonstration of proliferating cell nuclear antigen. Sections (3 µm) were cut, air dried at room temperature, dewaxed, and taken through serial solutions of alcohol. Endogenous peroxidase was blocked as described above, and the sections were incubated for two hours at room temperature in a 1:20 dilution of the PC10 monoclonal antibody. Visualisation was performed using a similar method as described above; diaminobenzidine-H₂O₂ was used as the chromagen and Cole's haematoxylin as the counterstain. The labelling index was determined by the

Figure 1: Immunohistochemical localisation of proliferating cells in FAP tissue using PC10 monoclonal antibody directed against proliferating cell nuclear antigen.

ratio of the number of labelled cells to the total number of cells within a crypt. The mean labelling index was determined from at least 10 crypts per section.³⁷

Results

MITOTIC INDEX

Counting at least 10 crypts per biopsy specimen from six patients, the mean (SEM) number of cells/crypt was 1034 (48.75) and the native mitotic index was 10.82 (1.40) cells/1000 cells (1.08 (0.14)%). The mean crypt cell production rate in the same six patients was 5.29 (1.18) cells/crypt/hour (range 1.55–11.09). Using this figure, a value for the birth rate of cells (K_b) can be calculated as $5.12 \text{ cells}/1000 \text{ cells}/\text{hour}$ – that is, $4.61/1034 \times 1000$.

PROLIFERATING CELL NUCLEAR ANTIGEN

Strong staining with the PC10 antibody was seen in histological sections from all six patients used to determine the mitotic index (Fig 1). The zone of proliferation extended from the crypt base to a point just over halfway up the crypt. To calculate the labelling index

and hence an approximation to the size of the proliferative compartment, clearly stained cells were counted. The mean (SEM) labelling index was 484 (40)/1000 cells (48.4 (4.0)%). Using this labelling index as an approximation of the growth fraction (I_p), then the cell cycle time (t_c) can be calculated³⁸ from the equation $t_c = I_p/K_b$, giving a value of 94.5 hours. In addition, the mean duration of mitosis (t_m)³⁸ can be determined from the equation $t_m = I_p/K_b \cdot I_m$, giving a value of 1.02 hours or 60 minutes.

CRYPT CELL PRODUCTION RATE (Fig 2)

The explants showed excellent preservation of tissue architecture after 18 hours of organ culture. The median control metaphase count was 11.72 cells/crypt (range 4.16–18.70) at one hour, 14.36 cells/crypt (range 5.11–33.46) at two hours, and 17.42 cells/crypt (13.22–35.87) at three hours. Epidermal growth factor (10 ng/ml) increased mean (SEM) rectal crypt cell production rate by 102% in paired biopsy specimens from 3.62 (0.59) to 7.33 (0.90) cells/crypt/hour ($n=8$, $p<0.01$). Calcipotriol ($1 \times 10^{-6} \text{ M}$) reduced mean rectal crypt cell production rate by 52% in paired specimens from 5.29 (1.18) to 2.56 (0.80) cells/crypt/hour ($n=6$, $p<0.05$). There was no significant difference between the post MC-903 crypt cell production rate value and the control crypt cell production rate value in the EGF group, and this might reflect the wide variation seen in the overall control crypt cell production rate values (range 1.55–11.09 cells/crypt/hour).

EGF RECEPTOR

Expression of the EGF receptor was seen in sections of control tissue taken from all eight patients who showed an increased crypt cell production rate with EGF in organ culture (Fig 3). EGF receptor expression occurred above the crypt base and increased in intensity further up the crypt to well above the mid-zone of the crypt. No expression was seen in any of the immunohistochemical negative controls.

Discussion

The active metabolite of vitamin D ($1,25 (\text{OH})_2 \text{D}_3$) has an inhibitory effect on in vitro cell proliferation (crypt cell production rate) in rectal tissue from patients with FAP.¹⁹ We have now shown that a synthetic analogue calcipotriol (with limited hypercalcaemic effects) produces a similar reduction of rectal crypt cell production rate in FAP tissue. If this effect could be reproduced in vivo, then vitamin D synthetic analogues might have a therapeutic role to control exuberant mucosal proliferation in the rectal stump of FAP patients. Although topical calcipotriol has a dramatic effect in psoriasis vulgaris,^{22–24} it has a very short half life and it is not available for enteric use.²¹ In conditions that are associated with an increased risk from colorectal cancer, such as ulcerative proctocolitis, there is a

Figure 2: The effect of epidermal growth factor (EGF) and calcipotriol (MC-903) on human rectal crypt cell production rate in FAP. Values are mean (SEM).

Figure 3: EGF receptor expression shown using the AP12E polyclonal antibody in a rectal crypt in tissue from a patient with FAP.

well reported increase in colorectal epithelial proliferation that persists even in quiescent disease.¹⁶ A similar dampening of colorectal epithelial proliferation might be beneficial in this inflammatory condition.

In addition to inhibition of proliferation, we have shown an ability to stimulate epithelial cell proliferation in FAP tissue using this in vitro technique. For the first time we have shown that epidermal growth factor can increase crypt cell production rate in human colorectal mucosa maintained in short term organ culture. We have also shown the expression of EGF receptor in control mucosal tissue from these patients. These findings correlate well with previously reported effects of EGF on the colorectum.^{25 26} As both sides of the biopsy specimen were bathed in culture medium, it cannot be determined whether this is a luminal or a humoral action of the peptide. The increased crypt cell production rate seen may be similar to the phenomena reported by Wright *et al.*^{27 28} Alternatively, it may represent an action of the peptide on an abnormal expression of EGF receptor in patients with FAP. We have not studied the effect of EGF on in vitro crypt cell production rate in tissue taken from normal controls and indeed, such a study could aid the interpretation of our results.

Using immunohistochemistry (PC10) in combination with static and dynamic parameters of cell proliferation (I_m and crypt cell production rate), we can estimate the time spent in various phases of the cell cycle. Proliferating cell nuclear antigen can be labelled with the PC10 antibody in formalin fixed paraffin wax embedded tissue.^{37 39} This antibody shows a higher labelling index when compared with the exclusive S phase marker bromodeoxyuridine,³⁷ and therefore it may give approximate figures for the growth fraction that are similar to previous published estimates⁴⁰ and it seems to be a reproducible and quantifiable marker of cell proliferation.³⁷ Because FAP tissue stains strongly with the PC10 antibody, proliferating cell nuclear antigen labelling can readily estimate the

growth fraction. If the cell birth rate is also known, the two figures can be used to estimate the cell cycle time. Our figure of about 95 hours is rather longer than estimates using thymidine labelling, which vary from 24–48 hours in colorectal cancers^{38 40 41} to 32 hours in patients with adenomas⁴² and 72–96 hours in the normal colon and rectum.^{38 43} Our estimates must, however, be viewed as provisional until validated by more standard methods of determining cell cycle times such as thymidine labelling. In addition, direct comparisons with previously reported values are not appropriate because our patients had previously undergone total colectomy. Also, PC10 may overestimate the growth fraction because of its long half life.^{37 39 44} Nevertheless, the combination of immunohistochemistry with static mitotic and dynamic metaphase counting might in the future give useful kinetic information about the gastrointestinal epithelium in disease states.

The authors wish to thank The Polyposis Registry at St Mark's Hospital for their help. Financial assistance for this work was provided by the Nutritional Consultative Panel.

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